U.S. ARMY MEDICAL RESEARCH INSTITUTE OF CHEMICAL DEFENSE

USAMRICD-TR-99-03

Use of the Comet Assay to Detect DNA Damage in Sulfur Mustard-exposed Human Peripheral Blood Lymphocytes

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July 1999

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REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188

1. AGENCY USE ONLY (*Leave blank*)	2. REPORT DATE July 1999	3. REPORT TYPE AND DATES COVERED 26 June 1997 - 16 December 1998

4. TITLE AND SUBTITLE Use of the Comet Assay to Detect DNA Damage in Sulfur Mustard-exposed Human Peripheral Blood Lymphocytes	5. FUNDING NUMBERS 30162384ATC2 62384A
6. AUTHOR(S) Moser, J., Levine, C.F., Thomas-Dunmeyer, D.R., Callan, A.J., and Smith, W.J.	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research Institute of Chemical Defense ATTN: MCMR-UV-PA 3100 Ricketts Point Road Aberdeen Proving Ground, MD 21010-5400	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research Institute of Chemical Defense ATTN: MCMR-UV-RC 3100 Ricketts Point Road Aberdeen Proving Ground, MD 21010-5400	10. SPONSORING/MONITORING AGENCY REPORT NUMBER USAMRICD-TR-99-03
11. SUPPLEMENTARY NOTES	
12a. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution unlimited	12b. DISTRIBUTION CODE

13. ABSTRACT (*Maximum 200 words*)

Sulfur mustard (HD) is a vesicating agent that alkylates cellular DNA and produces DNA strand breaks. The comet assay (single-cell gel electrophoresis) was used to detect HD-induced DNA single strand breaks in human peripheral blood lymphocytes (PBL). Specific steps taken to perform the comet assay in our laboratory are outlined. Briefly, PBL were mixed with agarose, mounted on a microscope slide, and lysed. The cellular DNA was treated with an alkaline buffer for denaturation, electrophoresed, and stained with ethidium bromide for visualization. Under a fluorescence microscope, cells with undamaged DNA appeared as intact comet heads without tails. Damaged DNA with single-strand breaks migrated in the direction of the anode, and this produced the appearance of a comet. The amount of DNA that migrates is proportional to the number of DNA single-strand breaks, as determined in past studies. Using an image analysis system, relative amounts of DNA damage were obtained by measuring the length of the comet tail and the intensity of fluorescence in the tail. The comet assay has the capacity to measure the ability of a compound to increase the rate of DNA repair in HD-damaged cells. Thus, it may potentially be used for rapid *in vitro* screening of antivesicant compounds.

14. SUBJECT TERMS comet assay, lymphocytes, sulfur mustard, vesicant	15. NUMBER OF PAGES 27
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UNLIMITED

PREFACE

The work reported herein was conducted under USAMRICD protocol 1-11-95-000-A-720, entitled "Development of Cell Cycling and DNA Repair *In Vitro* Assays for Evaluating Antivesicant Therapy." The data is recorded in USAMRICD notebooks 044-97, 045-97, and 039-98.

ACKNOWLEDGMENTS

The authors wish to thank Charlene Corun, Juanita Guzman, Eric Nealley, and Marian Nelson for technical assistance.

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INTRODUCTION

Sulfur mustard (HD) is a cytotoxic vesicant chemical warfare agent, or "blister agent," that produces severe lesions on exposed epithelial tissues. The most sensitive target of HD toxicity is cellular DNA. HD preferentially alkylates the N-7 position of guanine or the N-3 position of adenine, and alkylation leads to depurination of DNA strands. Subsequent breakage of phosphodiester bonds at the apurinic sites produces DNA single-strand breaks (reviewed in Papirmeister *et al.*, 1991). To repair DNA breaks, the resulting gap in the DNA chain is filled with appropriate bases (DNA polymerization), and these added bases are then ligated to complete the DNA strand repair.

The comet assay, also known as single-cell gel electrophoresis, is a fast and efficient method for detecting DNA single-strand breaks in individual cells (Singh *et al.*, 1988; de With *et al.*, 1994). This assay can be used to monitor the rate of DNA repair by measuring the disappearance of DNA single-strand breaks over time.

To perform the comet assay, nucleated cells are embedded in agarose gel on microscope slides and lysed by detergents at high salt concentrations. The cellular DNA is treated with a strong alkaline buffer to produce single-stranded DNA, electrophoresed under alkaline conditions, and stained with a fluorescent dye for visualization. When viewed with a fluorescence microscope, DNA of an undamaged cell appears as a spherical mass occupying the cavity formed by the lysed cell. When DNA is damaged, the negatively charged pieces of DNA are free to migrate in the electric field toward the anode and are seen streaming from the spherical mass (the comet head) in the shape of a comet tail (Kent *et al.*, 1995).

Both the length of the comet tail and the intensity of the fluorescence in the tail provide information about the number of DNA single-strand breaks in each cell. Single-stranded DNA migrate according to molecular length (Freeman *et al.*, 1986), with shorter DNA (that with more damage) migrating further from the comet head than longer DNA (that with less damage). However, free broken ends of the negatively charged DNA molecule may migrate only a short distance from the comet head, the DNA stretching from attachment sites on larger DNA pieces (Fairbairn *et al.*, 1995). Stretching of attached pieces of DNA, rather than free migration, is more likely to occur with a low amount of DNA damage. With increased damage, individual pieces of DNA are free to migrate into the comet tail. Thus, the product of comet tail length and intensity of the tail (comet moment) is the measurement used to describe the relative amount of DNA strand breakage in a cell, and it has been correlated quantitatively with the frequency of DNA strand breaks (Olive and Banath, 1993). Comet moment takes into account both damaged DNA that is free to migrate and damaged DNA that stretches into the comet tail from its attachment to larger DNA pieces.

The comet assay has several advantages (Tice *et al.*, 1990; de With *et al.*, 1994). Since DNA migration data are obtained in individual cells, this assay can provide information on the intercellular distribution of DNA damage and repair. The assay requires only a small number of cells for analysis, and virtually any eukaryotic cell population can be used. The comet assay is simple and fairly inexpensive. It is a fast and efficient method compared with conventional electrophoresis techniques, since DNA extraction is not required nor is enzymatic cleavage, a process by which information about strand breaks could be lost. Disadvantages of the comet assay are the necessity for

single cell suspensions; cells of an adherent cell culture may be damaged when harvested, offsetting results of DNA repair assays. Also, small cell samples may not represent the total cell population (Tice *et al.*, 1990).

The purpose of this study was to develop the comet assay to detect DNA single-strand breaks in HD-exposed human peripheral blood lymphocytes (PBL) and ultimately to use this assay for *in vitro* screening of potential antivesicant compounds. This report describes the comet assay procedure in detail.

MATERIALS AND METHODS

Cells. Human PBL were acquired from normal adult volunteers by the procedure outlined in human use protocol USAMRDC Log No. A-3575. Blood ($\leq$ 200 ml) was drawn by venipuncture, and the PBL were isolated from whole blood by differential centrifugation on a Percoll gradient (density = 1.080 g/ml at 20°C) as previously described (Meier *et al.*, 1987). Cells were washed twice with calcium-free and magnesium-free Tyrode's buffer and once with RPMI 1640 medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 0.05 mg gentamicin/ml RPMI 1640. Between each wash, the cells were centrifuged at 900 x g for 20 min at 20°C. The number of PBL obtained was determined by counting the cells on a hemocytometer. Following the final centrifugation, the isolated PBL were resuspended at 2×10^7 cells/ml in RPMI 1640 medium. The cells were then dispensed into 24-well tissue culture plates to yield a final concentration of $2\text{-}3 \times 10^6$ cells/well.

HD Treatment of Cells. Sulfur mustard with a purity of $\geq$ 98% was obtained from the Edgewood Research, Development and Engineering Center (Aberdeen Proving Ground, MD, USA). Stock solutions of HD were prepared as 4 mM solutions in RPMI 1640 medium.

PBL were exposed to HD concentrations ranging from 3 to 1000 µM by diluting stock HD in RPMI 1640 medium and immediately adding aliquots to PBL cultures (Meier and Johnson, 1992) in 24-well plates. Final volume was 1 ml/well.

To allow venting of volatile HD and permit its complete hydrolysis, control and exposed cells were incubated at room temperature (20 to 22°C) for 1 hour after exposure in a surety hood. At room temperature in aqueous media the half-life of HD is approximately 5 min, and HD hydrolysis is essentially complete by 1 hour (Cohen, 1946). Following room temperature incubation, the lymphocytes were transferred to a 37 °C, 5% CO_2-95% air incubator until harvested.

Harvesting PBL. PBL were harvested at various times up to 24 hours after HD exposure. After triturating several times, the PBL were suspended in RPMI 1640 medium and transferred from each well of the 24-well plate into individual 1.5 ml microcentrifuge tubes.

Viability Determination. After harvesting the cells, a 200 µl aliquot from each PBL sample was dispensed into a 12 x 75 mm test tube and diluted by the addition of 200 µl of RPMI 1640 medium to each tube. Then 50 µl of propidium iodide (Sigma Chemical

Company, St. Louis, MO, USA; dissolved in RPMI 1640 medium at 0.3 mg/ml) was added to each tube containing PBL. After a 2-5 min room temperature incubation in the dark, propidium iodide incorporation into the cells was assessed using a Fluorescence Activated Cell Sorter flow cytometer (Becton Dickinson, San Jose, CA, USA). The data were analyzed with the Lysis II computer software program (Becton Dickinson) and viability results were expressed as the percent of the total number of cells that excluded propidium iodide (Smith *et al.*, 1991; Clayson *et al.*, 1993).

Comet Assay. The comet assay was performed as described in Appendix A, and solutions used in the assay were prepared as described in Appendix B. Briefly, fully frosted microscope slides were covered with a base layer of 1% normal melting agarose. PBL were diluted in 1% low melting agarose, and this cell-agarose mixture was added as a second layer onto the microscope slide. A third layer of agarose was formed on the slide by the addition of 1% low melting agarose. The cells on the slide were lysed, and the DNA was denatured, electrophoresed, and then stained with ethidium bromide.

Image Analysis. The ethidium bromide-stained DNA was visualized as outlined in Appendix C, using a fluorescence microscope equipped with a 515-560 nm excitation filter and a 590 nm barrier filter, connected through a gated CCD camera to a computer-aided image analyzer. Imaging software was used for image analysis. Results were expressed as comet moment (the product of tail length and tail intensity).

Data Analysis. Data in the example given are summarized as mean ± SEM of comet moment. For each HD concentration, two slides were prepared, and 25 cells/slide were analyzed. A one-way analysis of variance (ANOVA) was used to determine whether there were significant differences in comet moment of cells exposed to different HD concentrations. A one-way ANOVA was also performed to assess whether there were significant differences in viability with varying HD concentrations. To determine whether the slide location in the electrophoresis unit significantly affected the comet moment measured, a three-way ANOVA was used to test for differences in rows, columns, and the HD concentrations studied for this determination (0, 60, 100, and 300 μM HD). Experiments were conducted at least three times with similar results.

RESULTS

As an example, photographic images of control PBL and PBL exposed to 100, 300, and 1000 μM HD, followed 4 hours later by preparation with the comet assay, are shown in Figure 3. These images represent the "live images" as they appear under a fluorescence microscope. Damaged cells appear like comets, with the tail length and the intensity of fluorescent staining in the tail dependent on the amount of DNA damage. Cells with more DNA damage displayed an increase in DNA migration from the nuclear region towards the anode and also displayed increased intensity in the tail.

In this example experiment, the appearance of comets differed depending upon the amount of HD to which the PBL were exposed. No migration of DNA occurred among the majority of the control cells. As HD concentration increased above 100 μM, there

was substantial increase in the amount of DNA in the tails as well as an increase in the length of migration. This was evident by changes that could be visually assessed in the comet images (Fig. 3) and by changes in the comet moment (Fig. 4). There were no significant differences in comet moment with HD concentrations ≤ 60 μM.

There was no significant loss in PBL viability at 4 hours after HD exposure. The viability remained above 90% at all HD concentrations tested (data not shown).

Problems encountered during the development of the comet assay and the steps taken to resolve these problems are outlined in Appendix D.

DISCUSSION

There is, to date, no specific strategy to treat or prevent HD-induced injuries. To develop prophylactic or therapeutic antivesicants, *in vitro* tests must be designed to quickly evaluate large numbers of candidate antivesicant compounds and identify those compounds with the highest potential for antivesicant activity. The comet assay is a rapid *in vitro* test that may provide useful information about the ability of a compound to either prevent DNA single-strand breaks or, by monitoring the disappearance of DNA single-strand breaks over time, enhance DNA repair in HD-damaged cells.

In assessing HD-induced DNA single strand breaks, there are limitations to the comet assay when performed as outlined. As described in Appendix D, Problem 9, the assay is useful in determining the relative amounts of DNA single strand breaks up to 6 hours following HD exposure. However, after 6 hours the cells appear to disintegrate in proportion to HD concentration and time following exposure, and the remaining cells do not represent the entire population.

The comet assay did not detect DNA damage in PBL at the low HD concentrations or early time points following exposure as reported using alternate methods (Meier and Millard, 1998). At low HD concentrations (in the example given, ≤ 60 μM HD at 4 hours post exposure), the amount of DNA damage detected with the comet assay was not significantly different from that of the control. This is presumably due to the formation of HD-induced DNA crosslinks. Assay modification to eliminate crosslinks is indicated if DNA damage produced at low concentrations of HD is to be evaluated.

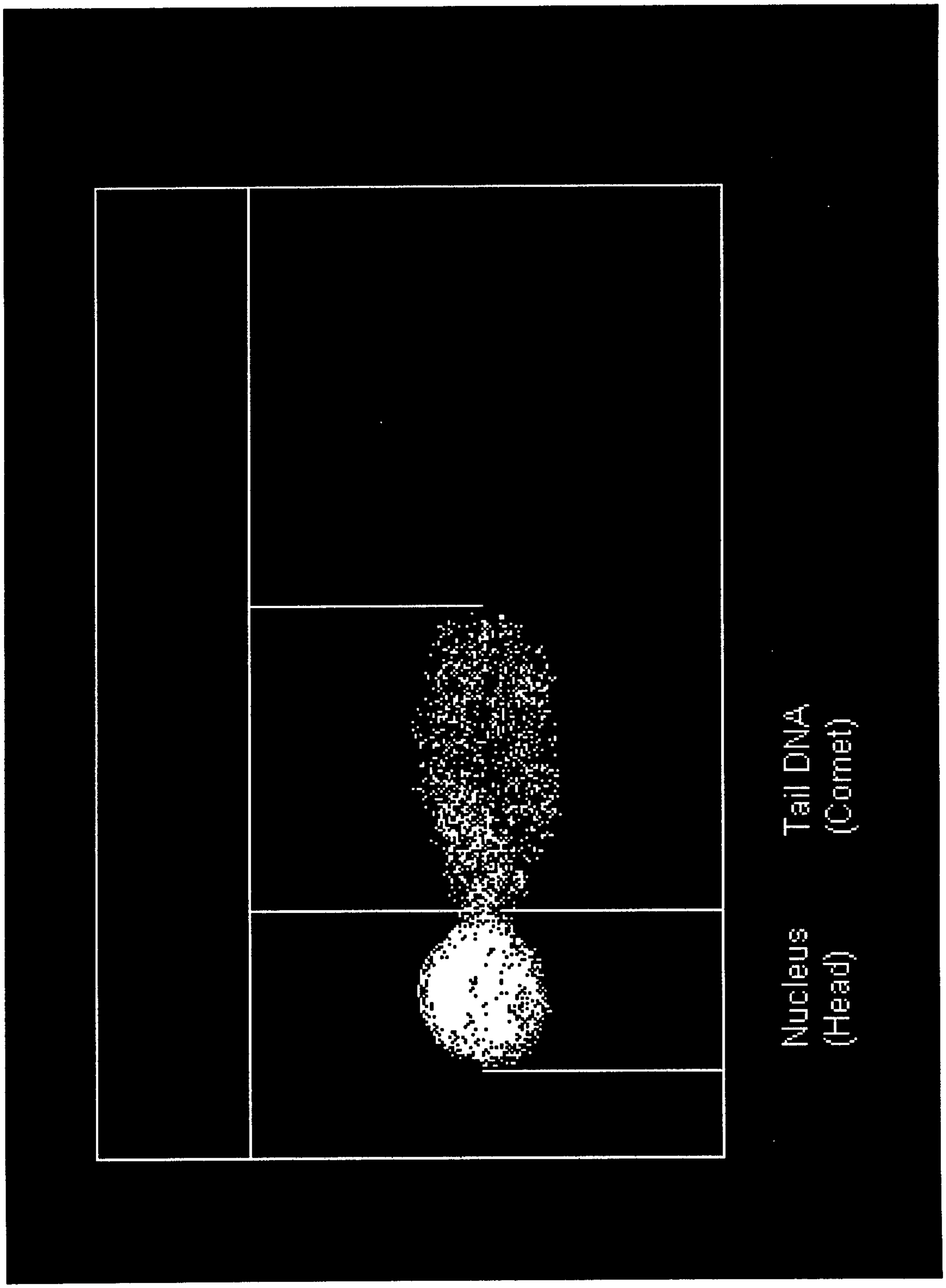

Figure 1. A schematic drawing of a comet image in a region of interest (ROI) box. Komet 3.1 computer software was used for image analysis.

		4 Hour Post HD Exposure			25 Cells/Slide	2 Replicates/Conc	Lengths:	Intensities:
Name	Notes	Cell Area	Head DNA	Tail DNA	L/H	TailExtentMoment	Tail Length	Comet Mode
MEAN	control	10839.4	94.6502	5.34979	0.985178	0.801833	11	288.747
ST.DEV.		14.6949	4.86663	4.86663	0.205847	1.92387	4.77324	12.8232
ST.ERR		1.83686	0.608329	0.608329	0.0257309	0.240484	0.596655	1.6029
MAX		10853	98.3306	39.9881	2.16667	15.9952	40	320
MIN		10763	60.0119	1.66941	0.769231	0.116859	7	251
MEAN	3 um HD	10826.2	94.6844	5.31565	0.985743	0.866671	13.0133	290.92
ST.DEV.		13.4152	3.96046	3.96046	0.274327	1.67572	5.25175	10.7876
ST.ERR		1.6769	0.495057	0.495057	0.0342909	0.209464	0.656469	1.34845
MAX		10840	98.4357	34.4147	2.83333	14.4542	42	329
MIN		10763	65.5853	1.56434	0.75	0.125147	8	266
MEAN	6 um HD	10826.4	95.5283	4.47171	0.991173	0.66038	13.48	286.4
ST.DEV.		12.1564	1.7528	1.7528	0.173919	0.491003	4.10129	9.73292
ST.ERR		1.51955	0.2191	0.2191	0.0217399	0.0613754	0.512661	1.21661
MAX		10843	99.3458	10.3797	1.65385	2.69871	30	308
MIN		10767	89.6203	0.654179	0.676471	0.0392507	6	267
MEAN	10 um HD	10825	95.3202	4.67981	1.00615	0.710929	13.1467	289.467
ST.DEV.		14.5448	3.36035	3.36035	0.180338	0.978203	3.96162	10.2038
ST.ERR		1.81811	0.420044	0.420044	0.0225423	0.122275	0.495202	1.27547
MAX		10841	98.6208	29.805	1.9	8.34539	28	312
MIN		10731	70.195	1.37922	0.733333	0.0965451	7	266
MEAN	30 um HD	10821.4	92.2631	7.7369	1.10473	1.10571	13.8933	293.52
ST.DEV.		13.5235	1.99834	1.99834	0.0933051	0.411353	2.09616	14.1214
ST.ERR		1.69044	0.249793	0.249793	0.0116631	0.0514191	0.26202	1.76518
MAX		10846	97.8516	13.0507	1.30769	2.74065	21	329
MIN		10786	86.9493	2.14836	0.807692	0.171869	8	265
MEAN	60 um HD	10818.8	93.1989	6.8011	1.08161	1.0099	13.9467	290.613
ST.DEV.		14.7788	2.27393	2.27393	0.143643	0.569622	3.34459	15.2961
ST.ERR		1.84735	0.284242	0.284242	0.0179553	0.0712027	0.418074	1.91201
MAX		10842	98.2649	13.5513	1.79167	4.20089	31	326
MIN		10779	86.4487	1.73506	0.75	0.138804	8	257
MEAN	100 um HD	10807.4	90.8049	9.19514	1.15476	1.58825	16.28	290.267
ST.DEV.		17.3173	3.35521	3.35521	0.154193	0.834502	3.2239	11.265
ST.ERR		2.16466	0.419402	0.419402	0.0192741	0.104313	0.402987	1.40813
MAX		10836	97.1537	18.4809	1.55	4.29609	25	313
MIN		10764	81.5191	2.84635	0.846154	0.256171	9	259
MEAN	300 um HD	10799.2	72.7131	27.2869	1.87514	7.95117	27.6	286.92
ST.DEV.		16.8033	8.26222	8.26222	0.407282	3.86024	5.63531	13.6568
ST.ERR		2.10042	1.03278	1.03278	0.0509103	0.48253	0.704414	1.70709
MAX		10831	92.2902	51.2134	3.5	24.5824	48	315
MIN		10751	48.7866	7.7098	1.04545	0.925175	12	251
MEAN	600 um HD	10781.9	57.195	42.805	2.67303	16.8507	38.72	284.933
ST.DEV.		29.6455	6.50794	6.50794	0.426458	4.3524	5.1005	11.2001
ST.ERR		3.70569	0.813493	0.813493	0.0533072	0.54405	0.637563	1.40001
MAX		10812	78.2725	55.3274	3.85714	25.2151	49	315
MIN		10552	44.6726	21.7275	1.63636	5.43187	25	259
MEAN	1000 um HD	10786.1	51.7638	48.2362	3.60325	21.8848	44.12	275.04
ST.DEV.		26.4029	8.91484	8.91484	0.932888	7.13489	8.28538	10.262
ST.ERR		3.30036	1.11436	1.11436	0.116611	0.891862	1.03567	1.28275
MAX		10846	90.6225	65.1356	6.9	38.1919	64	302
MIN		10736	34.8644	9.37749	1.07143	1.5004	16	252

Figure 2. A representative spreadsheet in Microsoft Excel of data from computer analysis of comet images. Comet moment is labeled as "TailExtentMoment."

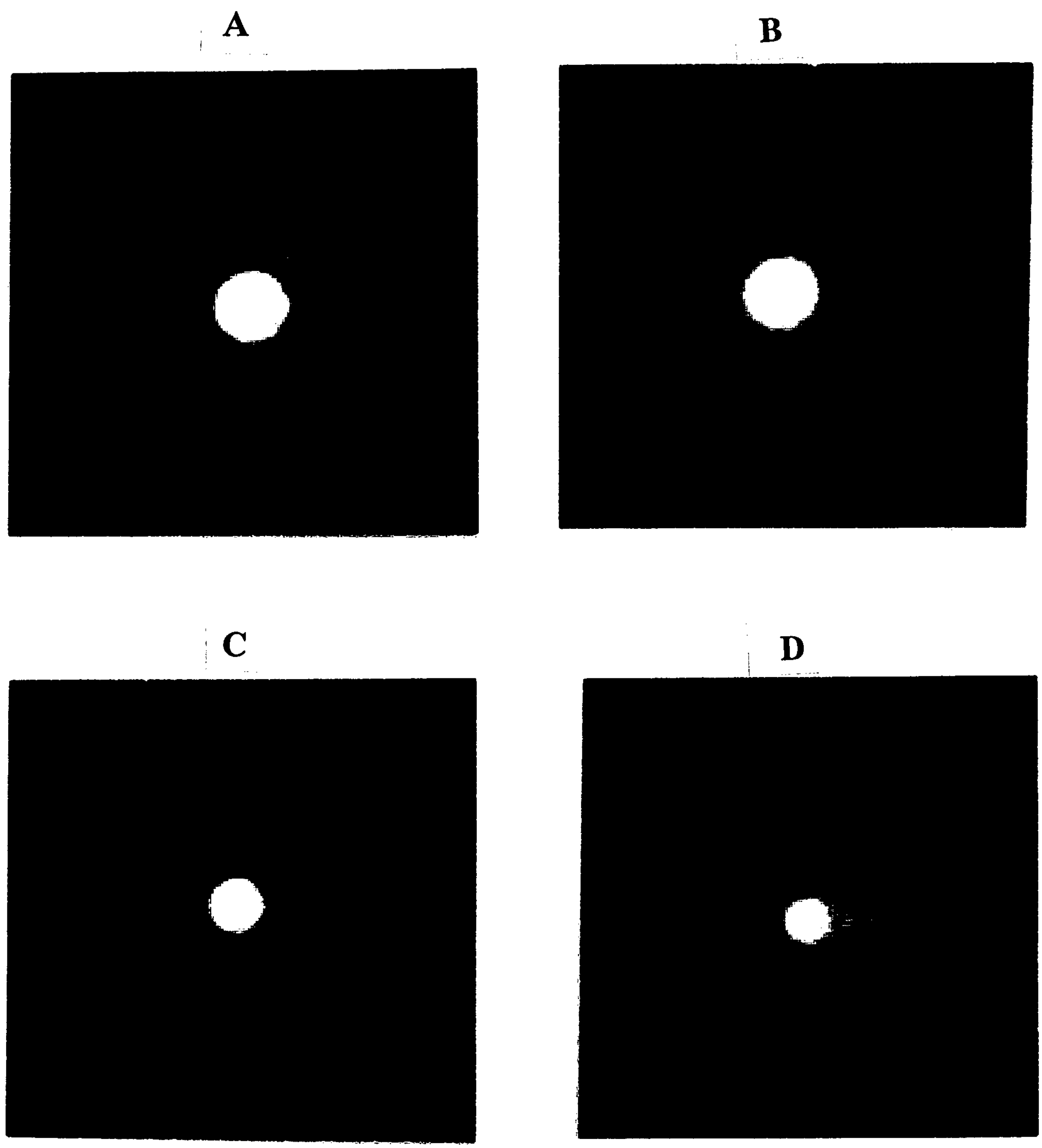

Figure 3. Photomicrographs of Comets from Cells Exposed to HD. PBL were exposed to A) buffer (control), B) buffer plus 100 μM HD, C) buffer plus 300 μM HD, or D) buffer plus 1000 μM HD. Cells were harvested at 4 hours after HD exposure and the comet assay was performed. The anode is to the right. Length and fluorescence intensity of the comet tail denote relative amount of DNA single strand breaks (longer length and higher intensity indicate more DNA damage). There appeared to be a HD concentration-dependent increase in DNA damage.

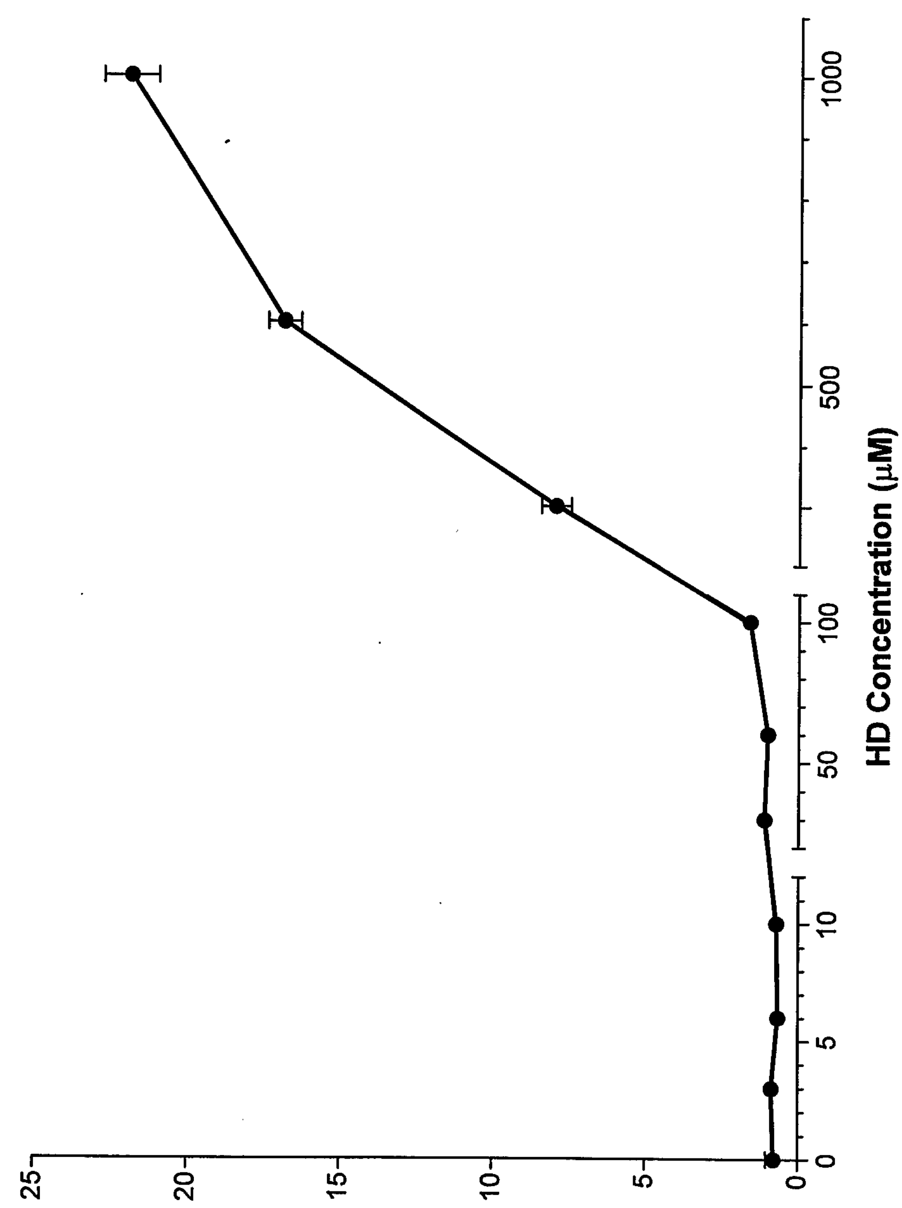

Figure 4. Effect of HD Concentration on Detectable DNA Damage. PBL were exposed to either buffer (control) or to buffer plus the indicated HD concentrations. Cells were harvested 4 hours after HD exposure and analyzed with the comet assay. For each HD concentration, two slides were prepared and 25 cells/slide were analyzed. There was an increase in DNA damage as HD concentration increased above 60 μM. Data are mean ± SEM of comet moment.

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APPENDIX A

Comet Assay

The following steps were performed to carry out the comet assay:

1. Fully frosted microscope slides (Fisher Scientific, Pittsburgh, PA, USA #12-544-5; 3" x 1" x 1mm), labeled at one end, were dipped into hot 1% NM agarose until 1/2 to 3/4 of each slide was covered. Excess agarose was drained from the slide, the unfrosted underside of the slide was wiped clean, and the slide was then completely air dried at room temperature.

Steps 2-6 were performed under dim or yellow light.

2. In a microcentrifuge tube, a sample of the cell suspension obtained from one well of the 24-well plate was diluted 1:10 in 1% LM agarose that had cooled to approximately 40 °C (e.g., 40 μl cells + 360 μl LM agarose). Seventy-five μl of the cell-agarose mixture was pipetted, as a second layer, onto the appropriately labeled microscope slide. The slide was quickly and carefully covered with a No. 1 coverslip (Corning, Inc., Horseheads, NY, USA #12531K; 24 x 60 mm) by dropping the coverslip directly onto the agarose to avoid bubble formation. These steps were repeated for each sample, completing all steps for one sample before starting the next. The agarose was allowed to solidify on the slides at room temperature for approximately 15 minutes.

3. After gently removing the coverslip, a third layer of agarose was formed by pipetting 100 μl of 1% LM agarose onto each slide and then immediately replacing the coverslip. The agarose was allowed to solidify at 4 °C for at least 5 minutes.

4. Once the agarose solidified, the coverslips were gently removed, and the slides were placed in flat rectangular plastic containers. To lyse the cells, the slides were submerged in cold lysis solution to which Triton X-100 had been added. The containers were sealed tightly and stored at 4 °C for a minimum of one hour and a maximum of 4 weeks.

5. The lysis solution was then poured out of each plastic container as the slides stuck to the bottom of the container. To denature the DNA, the slides were submerged in cold electrophoresis solution and incubated at 4 °C for exactly 40 minutes. The electrophoresis solution was then poured into a separate waste beaker to neutralize at a later time.

6. The microscope slides were placed randomly, side by side, in a horizontal electrophoresis chamber (Fisher Scientific, Fisher Biotech Recirculating Large Horizontal System #FB-SBR-2025) with descriptive information oriented towards the cathode. To prevent undesired variability, the slides were placed so as not to overlap the edge of the inside lip of the chamber, with a maximum of 24 slides in the chamber. The chamber was filled with cold electrophoresis solution until the solution was 3-4 mm above the slides. Electrophoresis proceeded for 40 minutes at 25 V and 300 mA

(Electrophoresis Power Supply, EPS 600; Pharmacia Biotech, Piscataway, NJ, USA). The current was adjusted to 300 mA by raising or lowering the level of electrophoresis solution.

7. Following electrophoresis, the slides were placed back in the plastic containers and rinsed four times with neutralization solution (1 ml solution/slide) for 5 minutes per rinse. Neutralization solution was drained out of the containers between each rinse. If desired, the slides were stored in neutralization solution in sealed containers at 4 °C for up to 1 week.

Electrophoresis solution was poured out of the chamber into waste beakers and carefully neutralized with sodium bicarbonate to a pH between 4 and 10. The chamber was rinsed with deionized distilled water to prevent corrosion.

8. After the final rinse or after storage, neutralization solution was drained from the slides. To stain DNA, 100 μl of 4 μg/ml ethidium bromide was pipetted onto each slide, and a new coverslip applied. After 5 minutes, the coverslips were removed and the slides were rinsed with a small amount of deionized distilled water by gently covering, rather than immersing, the slides with water. The solution was poured off of the slides into hazardous waste containers to store until filtration or turn-in. Two additional drops of 4 μg/ml ethidium bromide were then pipetted onto each slide and new coverslips applied. The slides were then ready for image analysis. The slides could be stored in air-tight containers for up to three days before analysis, if necessary.

APPENDIX B

Comet Assay Solution Preparation

The following solutions were prepared for use in the comet assay.

1. Lysis Solution (1% N-lauroylsarcosine, 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10). One liter was prepared, and the excess was stored for up to one month.

a. To prepare 1% N-lauroylsarcosine, 10 g N-lauroylsarcosine (Sigma Chemical Company, St. Louis, MO, USA #L 5125, minimum purity 94%) and 100 ml deionized distilled water were stirred together slowly, to prevent excess bubble formation, until clear. Due to the time required to dissolve, preparation of 1% N-lauroylsarcosine commenced at least one-half hour before use.

b. In a separate container, 146.1 g NaCl (Fluka BioChemika, Switzerland #71378), 37.2 g EDTA (Sigma #E 1644), 1.21 g Tris base (Sigma #T 4661), 6 g NaOH pellets (Sigma #S 0899), and 600 ml deionized distilled water were stirred together at medium speed. For all of the EDTA to go into solution, additional NaOH pellets were added until the solution became clear, and the pH was adjusted to 10.0.

c. The two solutions, prepared as described in a and b above, were combined, the volume brought to 1 liter with deionized distilled water, and the solution was stirred briefly. Lysis solution was stored at room temperature.

d. Approximately 2 hours before use, 1 ml Triton X-100 (Sigma #T 9284) per 100 ml of lysis solution was added to the required volume of lysis solution to produce 1% Triton X-100 in lysis solution. This solution was refrigerated (4°C) until used in the assay.

2. Phosphate Buffered Saline (PBS; 0.15 M NaCl, 3 mM Na_2HPO_4, pH 7.4). One liter was prepared, and the excess was stored for up to three weeks. Stirred together were 8.76 g NaCl, 0.426 g Na_2HPO_4 (Sigma #S 0876, dibasic, anhydrous), and 600 ml deionized distilled water, and the pH was adjusted to 7.4 with HCl. The volume was then brought to 1 liter with deionized distilled water, and the solution was mixed. PBS was stored at 4°C.

3. Electrophoresis Solution (300 mM NaOH, 1 mM EDTA, pH ≥ 13). Four liters were prepared for each assay. Forty-eight g NaOH pellets, 1.49 g EDTA, and deionized distilled water were brought to a fluid volume of 4 liters, stirred, and the pH was adjusted to ≥ 13 with NaOH, if necessary. Electrophoresis solution was stored at 4°C.

4. Neutralization Solution (0.4 M Tris, pH 7.5). One liter was prepared for each assay. Stirred together were 48.44 g Tris base and 600 ml deionized distilled water until the Tris went into solution. After adjusting the pH to 7.5 with HCl, the volume was brought to 1 liter with deionized distilled water. Neutralization solution was stored at 4°C.

5. Normal Melting (NM) Agarose (1%). Fifty milliliters were prepared for each assay. Type I NM agarose (Sigma #A 6013; low EEO) , 0.5 g, and 50 ml PBS were brought to a rapid boil on a hot plate only until the agarose dissolved into solution; further boiling was avoided to prevent evaporation. One percent NM agarose was used immediately after boiling.

6. Low Melting (LM) Agarose (1%). Fifty milliliters were prepared for each assay. LM agarose (Sea Plaque, FMC Bioproducts, Rockland, ME, USA, #50101), 0.5 g, and 50 ml PBS were boiled as described for 1% NM agarose. One percent LM agarose was placed in a 40 °C water bath until ready for use.

7. Ethidium Bromide (4 μg/ml). Ten milliliters were prepared and stored for use in several assays. Twenty μl of 2 mg/ml ethidium bromide (Sigma #E8751) and 10 ml deionized distilled water were mixed in a polypropylene centrifuge tube. The tube was wrapped with aluminum foil and stored at room temperature.

APPENDIX C

Image Analysis

The comet images were analyzed as follows:

1. The imaging equipment was turned on in the order listed and allowed to warm up for at least 5 minutes: main power supply (Stab. Arclamp Power Supply HBO 100, Ludl Electronic Products, Hawthorne, NY, USA), shutter driver (Uniblitz Model D122, Vincent Associates, Rochester, NY, USA), videoscope (Ludl Electronic Products), and the videoscope camera (Videoscope International, Sterling, VA, USA) that was mounted on a BH-2 microscope (Olympus America, Inc., Melville, NY, USA).

2. A Pentium-based desktop computer with 16 Mb RAM running Microsoft Windows 95, a videographics system board (IP-8/AT interface card, Matrox International, Mooers, NY, USA), Komet 3.1 computer software (Kinetic Imaging Ltd., Liverpool, UK), and two monitors was previously connected to the microscope through the CCD videoscope camera for visualization and analysis of cell images. The computer was turned on, and the Windows startup screen appeared on the first monitor.

3. Before beginning analysis, a new folder was created to store the analysis data, and it was named with the date the experiment was performed.

4. When the Komet 3.1 program was selected, a box with "USER NAME" appeared. A name was entered, followed by a click on "OK." The Comet Options screen then appeared. The values were set at:

Head Threshold%: 40	Tail Thrshld/1000: 15
Smoothing Value: 4	B'gnd Height: 20
Tail Break Length: 5	Saturation Limit: 10

Experimental Options allowed the user to input the number of cells per slide that would be analyzed, the number of treatment doses, and the number of replicate slides per dose. For example, the following values were set:

Cells/slide: 25	Number of doses: 8	Replicates: 3

The program kept track of the number of cells that had been analyzed and used this information to prompt the user when to change slides.
Under Operational Options, only "Fluorescence," "Auto Background," and "AutoExpress on Store" were selected. All of the options in the Comet Options window had limit ranges. The Komet 3.1 User Guide (Kinetic Imaging Ltd.) listed these ranges on pages 9.3 – 9.6. After choosing all of the desired options, OK was clicked.

5. Before using the Komet 3.1 system for the first time, the XY scale was calibrated to allow the computer to convert pixels into microns. This scale was created for each

microscope objective that would be used. An XY scale file called 20Xcal.sca was created for use with the 20X microscope objective. It was created using an object with gradations of known length (i.e., a hemocytometer) and following the directions in the Komet 3.1 User Guide on pages 5.10 – 5.14. Once the scale was created, it was chosen under the "FILE" menu. "Open XY scale file" and then "Option – Set Camera" were selected. Then the .sca file was found and opened. After the scale file was opened, the program automatically used this calibration each time it was turned on. If a different microscope objective were to be used, the appropriate .sca file would need to be opened. Several .sca files can be saved, one for each objective.

6. The settings for the microscope were as follows:
   a. For cells to be viewed on the computer monitor, the V.C.V.C bar on the right of the microscope was pulled out entirely. If the bar was pushed in, the cells could be viewed through the microscope eyepiece.
   b. The shutter door on the back arm of the microscope was pushed in on the right as far as it would go.
   c. The switch on the shutter driver had to be in the N.O. position for light to shine through the microscope slide.

7. While slides could be analyzed in the "Free" operating mode, "START EXPMNT" on the toolbar had to be selected for the software to count the cells, prompt the user when to change slides, and save the data together as an experiment. Once START EXPMNT was selected, the computer prompted the user to clear all results. Then the first slide was placed on the microscope stage, the switch on the shutter driver was moved from the N.C. to the N.O. position, and "LIVE" was selected on the tool bar. A live image of the microscope slide became visible on the second monitor. Cells were brought into focus with the adjustment knobs on the microscope.

8. For a cell to be analyzed, it had to be in a region of interest (ROI) box. If a ROI box was not already present on the screen, it was created by selecting "CELL" on the tool bar. The mouse was used to draw the box. The entire cell plus comet had to fit inside the box (Fig. 1).

9. Once the cell of choice was in the ROI box and brought into focus, "STORE" on the tool bar was selected. All the appropriate parameters of the comet were then automatically measured, including the diameter of the head, the length of the tail, and the intensities of the head and tail.

If the measurement lines drawn by the computer did not correspond to what was considered the actual head and tail of the comet, the comet was measured manually. First, "DELETE" was selected to delete the faulty measurement. Then "INTERACTIVE H + T" was selected. The cursor was then positioned over the right hand edge of the head, and the left mouse button was clicked. Then the cursor was positioned over the right hand end of the tail, and the right mouse button was clicked. The computer recorded the new data.

10. After the number of cells that was previously set in the Comet Options box had been analyzed, the computer prompted the user to change slides or doses as was appropriate. It also prompted the user to save the data in the folder that was created before beginning the analysis. After analyzing the last slide, the computer prompted the user to save the statistics. These files were saved with the extension .xld (e.g., stats.xld).

11. To access the statistics, Microsoft Excel was opened. Then "FILE" and "OPEN" were selected. The folder in which the data were saved was opened, and then the statistics file (e.g. stats.xld) was opened. A Text Import Wizard box appeared. Under "Original Data Type," "Delimited" was selected. The "File Origin" should be Windows (ANSI). "NEXT" was clicked, and, in the next box, "Tab" was selected. "NEXT" was clicked again. Under "Column Data Format," "General" was selected. If one or more of the columns of data were not needed (for example, "Olive Tail Moment"), that column was highlighted and "Do Not Import Column" selected. Finally, "Finish" was selected. The data then appeared in a Microsoft Excel spreadsheet and descriptive information about the experiment was added to the sheet (Fig. 2). To save this data, "Files of Type" and "Microsoft Excel Files" were selected. This saved the data with an .xls extension.

12. To save a comet image, "LIVE IMAGE," "STORE," "FILE," and then "SAVE IMAGE" were selected. The image file was given a descriptive name with the extension .bmp. This file was saved in a designated folder.

APPENDIX D

Problems with the Comet Assay and Their Resolutions

Problem 1. Agarose dislodged from the slide when the slide was immersed in lysis solution or electrophoresis solution.

Resolutions: To enhance adherence, a) fully frosted microscope slides were used, b) NM agarose was applied to the slide, and the slide was dried completely before adding LM agarose, c) NM agarose was applied to the slide by dipping rather than by pipetting, d) 1% was found to be the optimum concentration for LM agarose, e) the agarose was permitted to solidify at room temperature rather than at colder temperatures, and f) LM agarose obtained from FMC Bioproducts adhered better than other brands.

Problem 2. DNA migration for control cells was larger than expected, at times appearing similar to cells exposed to low concentrations of HD.

Resolutions: Procedural adjustments that improved results were a) the amount of time the slides were incubated in electrophoresis solution was increased from 20 minutes to 40 minutes, b) the time allowed for electrophoresis was increased from 20 minutes to 40 minutes, and c) the experiment was executed under dim or yellow lighting to prevent DNA single-strand breaks that result from exposure to fluorescent lights. In addition, lights were turned off in the hood during the 1-hour incubation period following HD exposure.

Note: It was found that the amount of basal DNA damage in control lymphocytes varied between donors. Also, DNA in HD-exposed cells may have migrated a shorter distance than controls because HD-induced DNA crosslinking inhibited DNA migration.

Problem 3. There was a significant difference ($p<.05$) in comet moment between rows of slides on the electrophoresis unit but not between columns. This was assessed by using cells exposed to either 0, 60, 100, or 300 μM HD. For each HD concentration, 32 slides were prepared and electrophoresis performed.

Resolution: Initially, the slides were placed in four rows and eight columns on the electrophoresis unit, with the slides on the first and last rows slightly overlapping the edge of the inside lip of the chamber. The number of rows of slides placed on the electrophoresis unit was subsequently reduced to three, with no slides overlapping the inside lip, and this resulted in no significant differences in comet moment between rows or columns of slides for each of the HD concentrations tested.

Problem 4. The comet tail was not visible or the nucleus was so bright that it masked the comet tail.

Resolution: Staining with too little or too much ethidium bromide led to the above problems, respectively. For the second application of ethidium bromide, first two drops were applied to the slide. If more ethidium bromide was needed for visibility of DNA, one drop at a time was applied until the comet tail was visible.

Note: Attempts to use pico green in place of ethidium bromide were unsuccessful because the stain photobleached within seconds when placed under the fluorescent light of the microscope. Visibility disappeared too quickly to allow complete slide analysis.

Problem 5. DNA fragments were seen as halos around the nucleus instead of in a comet formation.

Resolution: The fluorescent bulb of the microscope was out of alignment. Therefore, a representative from OPELCO aligned the bulb so that the light was shining perpendicularly instead of at an angle.

Note: When a new fluorescent bulb was installed on the microscope, the bulb was allowed to burn for one hour before analyzing slides.

Problem 6. Either very few or no lymphocytes were harvested from the 24-well plates.

Resolution: The media containing cells in each well was triturated several times to prevent the cells from sticking to the sides of the plastic wells. This resulted in recovery of 2-3 x 10^6 cells/well.

Problem 7. Error messages appeared during image analysis that pertained to adjusting the background/saturation limit: "Cell Region Exceeds Saturation Limit. Abandon Measurement. Reset Image Grab Conditions or Adjust Regions on Image" or "Cell Region Background Has Ø Signal."

Resolution: These error messages were an indication that the intensity was too high. Under the Comet heading, "Comet Options" was selected. Under the Operations heading, "STOP ON SATURATION" was deselected. However, if "STOP ON SATURATION" was already deselected, the gray scale was adjusted, using the Komet 3.1 User Guide as a reference.

Problem 8. Depending upon the power supply used for electrophoresis, the comet moment varied significantly under otherwise identical experimental conditions. An EC500-90 Power Supply (E-C Apparatus Corporation, Holbrook, NY, USA; 300 mA maximum), a PS 500XT DC Power Supply (Hoefer Scientific Instruments, San Francisco, CA, USA; 400 mA maximum), and an Electrophoresis Power Supply 600 (Pharmacia Biotech, Piscataway, NJ, USA; 400 mA maximum) were compared.

Resolution: The power supplies that reached up to 400 mA gave more reliable and consistent results when compared with the power supply that reached a maximum of 300 mA.

Problem 9. At > 6 hours, the number of cells appearing on the slides decreased and the amount of debris particles increased in a HD concentration- and time-dependent manner. For example, compared with 6 hours following a 10 μM HD exposure, at 24 hours the number of cells had decreased by approximately 50%; in samples exposed to ≥ 30 μM HD, only debris particles were present. At 24 hours, the cells that were present appeared largely undamaged.

Resolution: Cells appearing on the slides at > 6 hours after HD exposure do not appear to represent the overall effects of HD exposure on DNA damage. Therefore, the comet assay is useful in determining the relative amount of DNA damage only up to 6 hours following HD exposure.

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